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RESEARCH PAPER

# Phylogenetic relationships of 18 passerines based on Adenylate Kinase Intron 5 sequences

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**Abstract:** The 18 species of bird studied originally are known to belong to muscicapids, robins and sylviids of passerines, but some disputations are always present in their classification systems. In this experiment, phylogenetic relationships of 18 species of passerines were studied using Adenylate Kinase Intron 5 (AK5) sequences and DNA techniques. Through sequences analysis in comparison with each other, phylogenetic tree figures of 18 species of passerines were constructed using Neighbor-Joining (NJ) and Maximum-Parsimony (MP) methods . The results showed that sylviids should be listed as an independent family, while robins and flycatchers should be listed into Muscicapidae. Since the phylogenetic relationships between long-tailed tits and old world warblers are closer than that between long-tailed tits and parids, the long-tailed tits should be independent of paridae and be categorized into aegithalidae. Muscicapidae and Paridae are known to be two monophylitic families, but Sylviidae is not a monophyletic group. AK5 sequences had better efficacy in resolving close relationships of interspecies among intrageneric groups.

**Keywords:** molecular phylogeny; Adenylate Kinase Intron 5; passeriformes; monophyly

## Introduction

Passeriformes are composed of more than half of all living bird species. With intense adaptive radiations, the origin and evolution of Passeriformes have been studied for a long time (Chang et al. 1992). Since the debates have been continued on avian morphological classification systems, it is very necessary to use more exact and efficient methods to perfect avian systematics. Studies showed that molecular classification system can provide much information and solve some divergent problems in the avian systematics (Nei et al. 2002).

The explosion use of DNA techniques can provide a great deal of new data for avian systematists. A new avian classification system based on DNA hybridization techniques was founded by Sibley & Ahlquist (1990), in which Passeriformes was divided into two suborders: suboscines and oscines. The Restriction Fragment Length Polymorphism (RFLP) technique has been used in avian phylogenetics. In this technique, passerine mitochondrial genomes were found to be genetically polymorphic (Li et al. 2002). Three titmice species (*Parus bicolor*, *P. inornatus*, *P. wollweberi*) in the North America were studied by Gill and Slikas (1992) using RFLP, and they found that these species originated in the Pliocene or early Pleistocene. In addition, DNA sequence analysis has also been used in avian systematics, which provided more classification systematic information. Adenylate Kinase Intron 5 (AK5) may perform well in recovering relationships from interspecies to interfamily (Shapiro et al. 2001). In the present study, we focused the attention on the phylogeny of 18 species of passerines including muscicapids, robins and sylviids. The phylogenetic relationships were studied among 18 species of passerines using Adenylate Kinase Intron 5 (AK5) sequences (Shapiro et al. 2001). Compared with earlier classification systems, new insights were provided about their phylogeny.

## Material and methods

### Taxon sampling

Samples of 18 species of passerines were collected from the Mao'ershan Mountain, Heilongjiang Province, China (Table 1). AK5 sequences of *Falco femoralis* (AF307890) was downloaded from GenBank as outgroups.

### DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted using standard

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phenol-chloroform extractions. We obtained the primers for amplifying AK5 sequences from previous papers: AK5b+: 5'ATTGACGGCTACCCTCGCGAGGTG3', AK6c-:5'CACCCGCCCGCTGGTCTCTCC3' (Shapiro et al. 2001). The primers are located in the exon 5 and exon 6 of Adenylate Kinase gene. AK5 PCR mixtures contained 1.25-U Blend Taq DNA polymerase, 10×buffer of 5 μL, 2-mM $MgCL_2$, 200-μM dNTPs, 0.4 μM of each primer, and 100-500 ng of DNA in a final volume of 50 μL. Reaction mixtures were subjected to the following PCR cycling protocol: with an initial 10 min at 94°C, followed by 35 cycles of 92°C for 45 s, 56–60°C for 60 s, and 72°C for 60 s, and a final extension after the last cycle at 72°C for 5 min. PCR products were purified with QIAquick PCR purification kit (Qiagen). Most of PCR products were sequenced directly and few of them were sequenced after cloning. Sequencing was performed by the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (ABI) and analyzed on an automated ABI sequencer.

**Table 1. Species examined in the present study**

| Taxon（Zheng Zuo-Xin 2002） | Academic name | Name code | Sample number | Accession numbers |
|---|---|---|---|---|
| Muscicapidae Turdinae | *Luscinia cyane* | Lcy | 2 | DQ119493 DQ119494 |
| Muscicapidae Turdinae | *Tarsiger cyanurus* | Tcy | 2 | DQ119499 DQ119500 |
| Muscicapidae Muscicapinae | *Ficedula mugimaki* | Fmu | 2 | DQ365012 DQ365013 |
| Muscicapidae Muscicapinae | *Ficedula zanthopygia* | Fza | 1 | DQ365014 |
| Muscicapidae Muscicapinae | *Cyanoptila cyanomelana* | Ccy | 1 | DQ365015 |
| Muscicapidae Sylviinae | *Phylloscopus trochiloides* | Ptr | 3 | DQ119518 DQ119519 DQ119520 |
| Muscicapidae Sylviinae | *Phylloscopus fuscatus* | Pfu | 1 | DQ119511 |
| Muscicapidae Sylviinae | *Locustella lanceolata* | Lla | 2 | DQ119495 DQ119496 |
| Muscicapidae Sylviinae | *Phylloscopus proregulus* | Ppr | 3 | DQ119512 DQ119513 DQ119514 |
| Muscicapidae Sylviinae | *Phylloscopus inornatus* | Pin | 3 | DQ119515 DQ119516 DQ119517 |
| Sittidae | *Sitta europaea* | Seu | 1 | DQ119498 |
| Muscicapidae Timaliinae | *Paradoxornis webbianus* | Pwe | 1 | DQ119497 |
| Hirundinidae | *Hirundo rustica* | Hru | 1 | DQ119492 |
| Paridae | *Aegithalos caudatus* | Aca | 2 | DQ116460 DQ119491 |
| Paridae | *Parus major* | Pma | 3 | DQ119505 DQ119506 DQ119507 |
| Paridae | *Parus ater* | Pat | 2 | DQ119503 DQ119504 |
| Paridae | *Parus palustris* | Ppa | 3 | DQ119508 DQ119509 DQ119510 |
| Paridae | *Parus montanus* | Pamo | 2 | DQ119501 DQ119502 |

### Data analysis

Sequences were proofed with DNAStar software package and performed similarity searching with Blast in the NCBI. Alignments were made with Clustalx (Hall 2001)（Version 1.8）and further analyses were conducted with Mega 3.0.

Phylogenetic analyses were first undertaken using Neighbor-Joining (NJ), and conducted with Phylip. The data were also analyzed using the Maximum-Parsimony (MP) method. Kimura Two-Parameter Model was used for base substitution analysis. Transitions and transversions were all included and all three substitution sites were used. Substitution rates of three sites were assumed to be different, which followed the Γ distribution with its shape coefficient of 0.5. Phylogenetic trees were analyzed by 1000 bootstrap replicates.

## Results

### Sequence analysis and base composition

With insertions and deletions, AK5 sequences differ in length from 451 to 530bp. AK5 genes of all species in this study have the GT and AG doublet sites that mark the beginning and end of the intron (Breathnach et al. 1977; Mount 1982; Keller et al. 1985; Mount et al. 1992). We obtained 35 AK5 complete sequences, which were deposited into Genbank. The accession numbers are as follows: DQ116460, DQ119491–DQ119520 and DQ365012–DQ365015.

The average contents of T, C, A and G in the 35 AK5 sequences were 16.8%, 30.9%, 22.4%, and 29.9%, respectively. The percentages of conserved sites, variable and parsimony-informative sites were 63.4%, 36.6% and 22.7%, respectively.

### Indels (insertions/deletions)

Considerable length variations were found among the AK5 sequences examined. The sequences contain numerous indels, which involve both single and multiple nucleotides. Most indels are from single evolutionary events (i.e., insertion or deletion of a single nucleotide or string of nucleotides). For example, there were four deletions shared by the *P. major*, *P. ater*, *P. palustris* and *P. montanus* (nucleotide sites: 22–36, 397–406, 417–441, 444–469). Consequently, these four species have the shortest AK5 sequences. There were one unique 5bp insertion (CCTAT, nucleotide sites 32–36) and one 1bp insertions (G, nucleotide sites 523) in the AK5 sequences of *P. webbianus* and *H. rustica*, respectively. It is believed that these indels represent unique evolutionary events of unequivocal homology, with no reversal.

Genetic distances

Genetic distances (Table 2) were calculated with Kimura Two-Parameter Model. The average genetic distance based on AK5 sequences among 18 species of passerines is 0.086. The genetic distance between *Tarsiger cyanurus* and *Luscinia cyane* was the smallest (0.045) and the largest (0.162) appeared between *Sitta europaea* and *Luscinia cyane*. The genetic distances between *Sitta europaea* and other old world warblers were relatively smaller than that between *Sitta europaea* and other passerines, which means that *Sitta europaea* has a relatively close relationship with old world warblers. The genetic distance between long-tailed tits and old world warblers was smaller than that between long-tailed tits and parids also, which means that old world warblers have closer relationship with long-tailed tits.

**Table 2. Pairwise distances between AK5 sequences of 18 species of passerines computed by Kimura Two-parameter model** (The lower triangle numbers are pairwise distances and the upper triangle standard error)

| Species | Fmu | Fza | Lcy | Tcy | Ccy | Aca | Ptr | Pin | Ppr | Pfu | Seu | Pwe | Hru | Lla | Ppa | Pamo | Pat | Pma |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fmu | | 0.008 | 0.013 | 0.010 | 0.012 | 0.024 | 0.020 | 0.022 | 0.020 | 0.020 | 0.023 | 0.019 | 0.022 | 0.022 | 0.017 | 0.017 | 0.017 | 0.018 |
| Fza | 0.026 | | 0.014 | 0.012 | 0.014 | 0.025 | 0.022 | 0.023 | 0.022 | 0.022 | 0.024 | 0.020 | 0.023 | 0.024 | 0.017 | 0.017 | 0.018 | 0.019 |
| Lcy | 0.051 | 0.064 | | 0.013 | 0.016 | 0.026 | 0.022 | 0.024 | 0.022 | 0.021 | 0.025 | 0.021 | 0.024 | 0.024 | 0.020 | 0.019 | 0.020 | 0.021 |
| Tcy | 0.034 | 0.045 | 0.054 | | 0.013 | 0.024 | 0.020 | 0.021 | 0.020 | 0.020 | 0.022 | 0.019 | 0.021 | 0.022 | 0.016 | 0.015 | 0.017 | 0.018 |
| Ccy | 0.046 | 0.058 | 0.073 | 0.054 | | 0.029 | 0.024 | 0.026 | 0.023 | 0.024 | 0.026 | 0.023 | 0.026 | 0.027 | 0.021 | 0.020 | 0.021 | 0.022 |
| Aca | 0.144 | 0.147 | 0.159 | 0.139 | 0.176 | | 0.013 | 0.014 | 0.013 | 0.013 | 0.018 | 0.013 | 0.016 | 0.018 | 0.018 | 0.018 | 0.016 | 0.017 |
| Ptr | 0.111 | 0.122 | 0.126 | 0.107 | 0.141 | 0.057 | | 0.004 | 0.005 | 0.008 | 0.014 | 0.012 | 0.015 | 0.016 | 0.015 | 0.014 | 0.013 | 0.014 |
| Pin | 0.122 | 0.134 | 0.137 | 0.118 | 0.153 | 0.060 | 0.008 | | 0.007 | 0.009 | 0.015 | 0.013 | 0.016 | 0.017 | 0.016 | 0.015 | 0.014 | 0.015 |
| Ppr | 0.111 | 0.122 | 0.126 | 0.107 | 0.134 | 0.057 | 0.010 | 0.018 | | 0.008 | 0.014 | 0.012 | 0.015 | 0.016 | 0.015 | 0.014 | 0.013 | 0.014 |
| Pfu | 0.111 | 0.122 | 0.118 | 0.107 | 0.141 | 0.057 | 0.021 | 0.029 | 0.021 | | 0.014 | 0.012 | 0.014 | 0.016 | 0.015 | 0.014 | 0.013 | 0.014 |
| Seu | 0.134 | 0.143 | 0.153 | 0.127 | 0.162 | 0.094 | 0.060 | 0.069 | 0.060 | 0.066 | | 0.015 | 0.017 | 0.019 | 0.017 | 0.016 | 0.015 | 0.016 |
| Pwe | 0.106 | 0.112 | 0.119 | 0.102 | 0.134 | 0.053 | 0.048 | 0.057 | 0.048 | 0.048 | 0.071 | | 0.013 | 0.014 | 0.014 | 0.013 | 0.012 | 0.013 |
| Hru | 0.127 | 0.135 | 0.142 | 0.123 | 0.158 | 0.078 | 0.066 | 0.076 | 0.066 | 0.060 | 0.084 | 0.056 | | 0.017 | 0.015 | 0.015 | 0.014 | 0.015 |
| Lla | 0.128 | 0.139 | 0.143 | 0.131 | 0.167 | 0.092 | 0.076 | 0.086 | 0.076 | 0.076 | 0.101 | 0.062 | 0.081 | | 0.017 | 0.016 | 0.015 | 0.016 |
| Ppa | 0.087 | 0.084 | 0.107 | 0.077 | 0.115 | 0.093 | 0.064 | 0.074 | 0.064 | 0.064 | 0.082 | 0.062 | 0.072 | 0.082 | | 0.004 | 0.007 | 0.008 |
| Pamo | 0.084 | 0.087 | 0.103 | 0.074 | 0.111 | 0.089 | 0.061 | 0.070 | 0.061 | 0.061 | 0.078 | 0.059 | 0.069 | 0.078 | 0.008 | | 0.006 | 0.007 |
| Pat | 0.090 | 0.094 | 0.110 | 0.087 | 0.118 | 0.079 | 0.055 | 0.064 | 0.055 | 0.055 | 0.072 | 0.048 | 0.063 | 0.072 | 0.018 | 0.015 | | 0.006 |
| Pma | 0.097 | 0.101 | 0.117 | 0.093 | 0.125 | 0.082 | 0.060 | 0.070 | 0.060 | 0.060 | 0.078 | 0.056 | 0.069 | 0.078 | 0.023 | 0.020 | 0.015 | |

Phylogenetic analyses

Phylogenetic trees of the 18 species of passerines were constructed by the NJ and MP methods (Fig. 1). The results showed that the two phylogenetic tree figures were not similar completely, but they all comprise three major clades. The first major clade was made up of old world warblers, long-tailed tits, crow-tits, barn swallow and sittids. The second was parids and the third was robins and flycatchers.

The clusterings of the 18 passerine species are approximately alike in the two tree figures with slight differences. In the first major clade, *Phylloscopus inornatus* and *Phylloscopus trochiloides* were clustered together, then clustered with *Phylloscopus proregulus* and then with *Phylloscopus fuscatus*. *Locustella lanceolata* was clustered with *Paradoxornis webbianu*. In the NJ tree figure, *Aegithalos caudatus* was clustered with the four warblers above, then with *Sitta europaea*. But in the MP tree figure, *Sitta europaea* was clustered with *Hirundo rustica*, then with *Aegithalos caudatus*. In the second major clade, *Parus palustris* was clustered with *Parus montanus* forming one small clade. In the NJ tree, *Parus major* was clustered with *Parus ater* to formanother small clade, but in the MP tree figure, *Parus major* was not clustered with *Parus ater*. In the third major clade, *Ficedula mugimaki* was clustered with *Ficedula zanthopygia*, and then with *Cyanoptila cyanomelana* to form a clade. This clade then clusters with *Luscinia cyane* and finally with *Tarsiger cyanurus* in the MP tree figure, but it clusters with *Luscinia cyane* first, then with *Tarsiger cyanurus* (Fig. 1).

## Discussion

Phylogenetic utility

From previous researches, nuclear introns were mainly used to reveal medium relationships between intergeneric and interfamilies. Russello and Amato (2004). studied the phylogenetic relationships of *Amozana*, using three nuclear introns and three mitochondrial gene sequences. The results showed that nuclear introns could also be used to analyze intrageneric relationships. A later study showed that intron sequences could also be used to analyze phylogenetic relationships of closely related species (Slade et al. 1994). As to AK5 sequences, it may perform well in recovering relationships among intermediate to distantly related congeneric species and between genera (Shapiro et al. 2001). Our results showed that *Paris poultries* and *Paris Montanez* were difficultly distinguished on morphological traits but easily distinguished with analysis of AK5 sequences. Therefore, AK5

sequences also have fine effectiveness in analyzing relationships among close related congeneric species.

Phylogenetic relationships

With regard to relationships among muscicapids, turdids and sylviids, three main opinions are coexisting. First, muscicapids, turdids and sylviids should be listed into three subfamilies (Muscicapinae, Turdinae and Sylviinae) under the family Muscicapidae (Zheng Zuoxin 2002); Second, muscicapids and turdids are listed into two subfamilies (Muscicapinae and Turdinae) under the family Muscicapidae, but sylviids is listed into the family Sylviidae (Sibley et al. 1990), Third, muscicapids, turdids and sylviids are all categorized as three families (Muscicapidae, Turdidae and Sylviidae), (Zheng Guangmei 2002; Howard et al. 2003). It should be pointed out that robins are isolated from Turdidae and listed into Saxicolinae under Muscicapidae in some classification systems (Sibley et al. 1990; Howard et al. 2003), but in others, they are still lying in Turdidae (Zheng Zuoxin 2002; Zheng Guangmei 2002). Due to the absence of other species of Turdidae in our research, we are not sure whether robins should be listed into Saxicolinae, which is independent of Turdidae or not. According to the phylogenetic analyses, flycatchers and robins have a close relationship, but sylviids are relatively far away from them, which basically supports the second opinion.

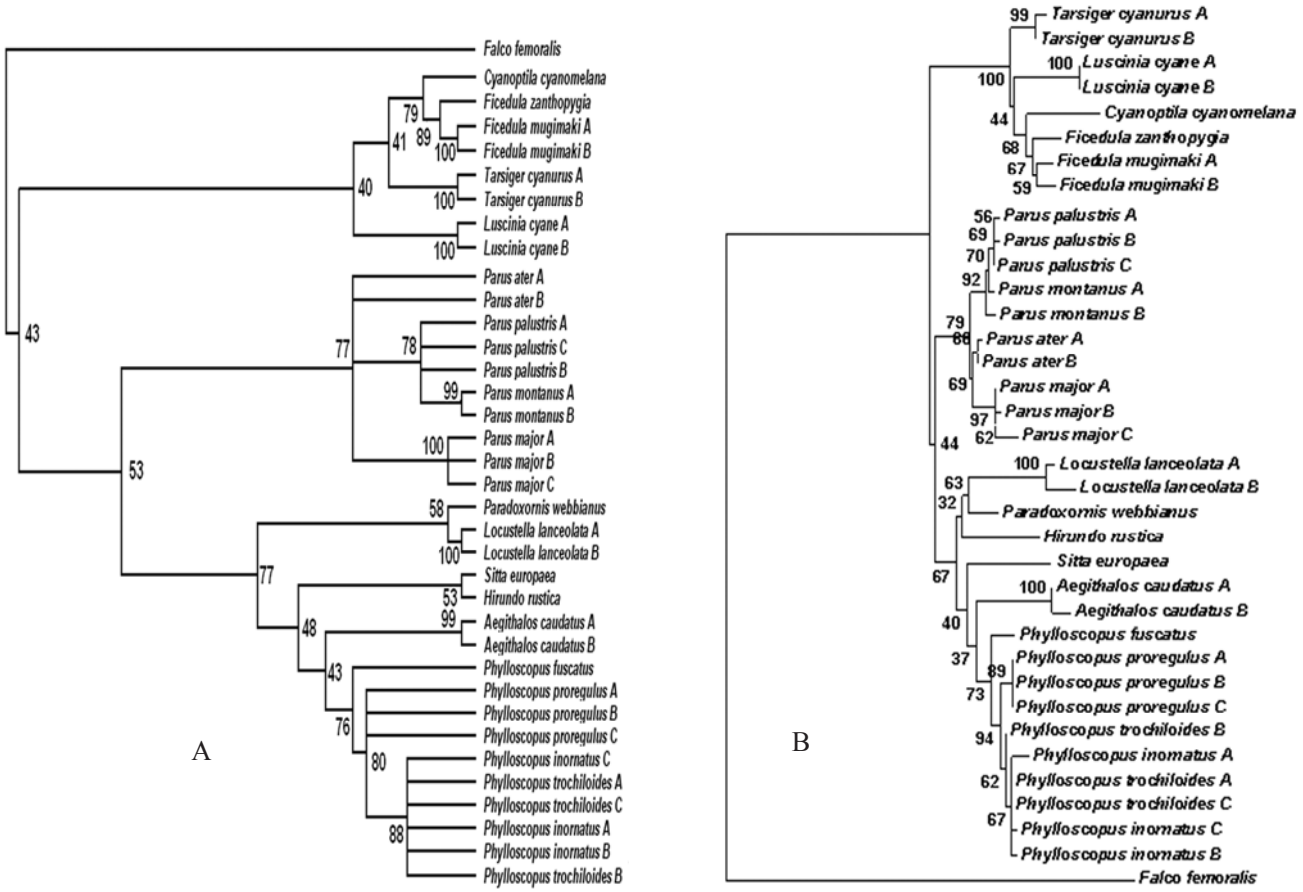


**Fig. 1 Phylogenetic tree figure of 18 passerine species constructed with AK5 sequences**

(A: NJ tree; B: MP tree)

In the classification system of Sibley & Ahlquist (1990), parids belong to the superfamily Sylvioidea. The result reveals that parids have close relationship with sylviids. Alstrom et al. (2006) studied the systematic status and the phylogenetic relationships of superfamily Sylvioidea, by combining mitochondrial Cytb and myoglobin intron Ⅱ sequences, and they reported that parids were excluded from superfamily Sylvioidea. Ttheir conclusions need to be examined further due to having less sample's kinds and amount of parids. In our study, the results from genetic distance between parids and sylviids showed that parids and sylviids had likely closer relationship. From phylogenetic trees constructed by two methods, parids always gathers sylviids first, so our research support Sibley & Ahlquist's classifying viewpoint.

In the classification system of Sibley & Ahlquist (1990), Sittidae belongs to the superfamily Sylvioidea. But in the results of Sheldon & Gill (1996), Sittidae was close to Sturnidae and Turdidae of the superfamily Muscicapoidea (Sheldon et al. 1996). Our results support the opinion of Sibley & Ahlquist, but further study should be conducted in the future.

In the NJ tree figure, *Hirundo rustica* clusters with *Locustella lanceolata* and *Paradoxornis webbianus* with low bootstrap

value (only 32%). In the MP tree figure, warblers first cluster with *Hirundo rustica* and then with *Locustella lanceolata* with high bootstrap value, which means that the relationship between *Hirundo rustica* and warblers has a closer relationship than that between *Locustella lanceolata* and warblers. Alstrom et al. (2006) also supported the opinion having a closer relationship between *Hirundo rustica* and warblers.

In the classification system of Sibley & Ahlquist (1990), the taxonomic position of crowtits is in Timaliini, Sylviinae, Sylviidae, Sylvioidea, however, in the classification system of Zheng Zuoxin(2002), crowtits are in Timaliinae and Muscicapidae. In another classification system, crowtits turn out to be Paradoxornithidae (Zheng Guangmei 2002). In our results, *Paradoxornis webbianus* clusters with *Locustella lanceolata*, which supports the opinion of Sibley & Ahlquist.

In the classification system of (Zheng Zuoxin 2002), long-tailed tits just belong to a genus under paridae. In the classification systems of Sibley & Ahlquist (1990) and Sheldon & Gill (1996), long-tailed tits are listed in an independent family of aegithalidae under Sylvioidea. Spicer et al. (2004) studied the phylogenetic relationships of Sylvioidea using 16SrRNA sequences, whose results supported the opinions of Sibley & Ahlquist (1990) and Sheldon & Gill (1996). Our results also supported the opinion that long-tailed tits should be independent of paridae and be categorized into aegithalidae. Also in our results, the relationships between long-tailed tits and old world warblers had closer relationship than that between long-tailed tits and parids. This may be a consequence of the relatively sparse taxon samping in the data set, and more and more scholars emphasized denseness sampling and increasing sample quantity (Omland et al. 1999; Rannala et al. 1998). The bootstrap numbers of long-tailed tits and sylvioidea cluster in phylogenetic trees constructed by amphi-method are all lower, so long-tailed tits' concrete position needs to be future studied.

From the phylogenetic tree figure and genetic distance, there are also disputes in intrafamily relationship. In Sylviidae, some species from genus warbler cluster together, it seems that the relationship between warblers and lanceolated warbler is farther than that between warblers and long-tailed tits.

Alstrom et al. (2006) concluded that the relationship between warbler and long-tailed tits was closer than that between warbler and lanceolated warbler, which is agreed with our conclusion.

Monophyly of group

The monophyly of the passerine is also remarkable all the time. Sibley & Ahlquist's (1990) taxonomic system revealed that superfamily Sylvioidea, Passeriodea and Muscicapoidea were all in monophyly. Sheldon & Gill (1996) thought that superfamily Sylvioidea and Passeriodea were not in monophyletic group, but superfamily Muscicapoidea was. Barker et al. (2002) thought that Sibley& Ahlquist's superfamily Sylvioidea, Passeriodea and Muscicapoidea were not in monophyly. From phylogenetic tree figure, Muscicapidae constructs one monophyly, but robins and flycatchers do not construct monophyletic group respectively. Paridae constructs one monophyly, whereas *Sitta europaea, irundo rustica*, *Paradoxornis webbianus* and the species in Sylviidae are all together. Therefore, Sylviidae is not one monophyletic group.

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